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PRINCIPAL INVESTIGATOR: Rebecca Lock

CONTRACTING ORGANIZATION: University of California
San Francisco CA 94143-0962

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Email: rebecca.lock@ucsf.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT **Subject:** During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. To date we have determined that autophagy is induced in breast epithelial cells expressing oncogenes that activate the Ras and PI3K pathways, and that autophagy inhibition slows the growth and invasive properties of transformed breast epithelial cells during 3D morphogenesis.

15. SUBJECT TERMS
autophagy, oncogenes, extracellular matrix detachment, 3D morphogenesis, cell transformation

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ANNUAL STATUS REPORT: BC083204. Detachment-Induced Autophagy and Breast Cancer Cell Survival

INTRODUCTION: **Subject:** During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. To date we have determined that autophagy is induced in breast epithelial cells expressing oncogenes that activate the Ras and PI3K pathways, and that autophagy inhibition slows the growth and invasive properties of transformed breast epithelial cells during 3D morphogenesis.

BODY: Below is a summary of results from tasks outlined in the approved Statement of Work that have been completed to date.

Task 1: *Determine if breast epithelial cells expressing activated PI3K and HER2/NEU induce autophagy during ECM detachment.*

A and C: *Examine LC3-I and II levels by western blot to monitor autophagy induction in MCF10A cells expressing PI3KH1047R and NeuT following suspension. C: Asses LC3 turnover by treating suspended cells with E64d and pepstatin A during suspension and monitor turnover by western blot:*

We have established MCF10A cell lines expressing the following oncogenes: PI3KH1047R, NeuT, and RasV12. These cells along with MCF10A cells expressing an empty vector as a control were plated on poly-hema coated 6-well dishes to prevent cell attachment. Cells were grown in suspension for 24 hours and treated with E64d and pepstatin A (lysosomal inhibitors) five hours before lysis. To examine baseline autophagy levels in attached conditions the same cells were also plated on uncoated 6-well plates, allowed to adhere, and treated with lysosomal inhibitors as described. Both adherent and suspended cells were lysed after 24 hours in RIPA lysis buffer and autophagy levels were examined by western blot using an antibody against LC3 (ATG8). We found that all the cell lines evaluated induced autophagy following suspension but to varying degrees when compared to empty vector control cells (Figure1). PI3KH1047R expressing MCF10A cells showed indistinguishable levels of LC3-II induction following suspension compared to vector control cells and the levels LC3-II in PI3KH1047R

FIGURE 1

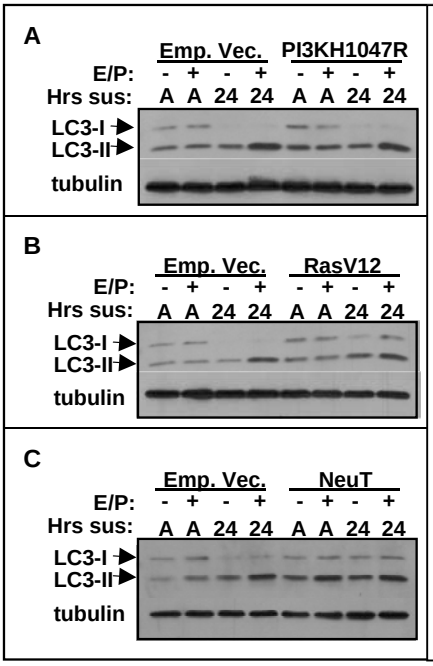

Figure 1: Levels of LC3-II and LC3-II in MCF10A cell expressing the indicated oncogene in attached (A) or following 24hrs of matrix detachment (24). Cell were either treated (+) or untreated (-) with E64d and pepstatin A (E/P) before lysis. **1A:** Empty vector control (Emp. Vec.) and PI3KH1047R MCF10As. **1B:** Empty vector control and RasV12 expressing MCF10As. **1C:** Empty vector control and NeuT expressing MCF10As.

were further increased upon addition of the lysosomal inhibitors (Figure 1A). We found that MCF10A cells expressing RasV12 also induce autophagy following suspension, but the induction levels were slightly reduced compared to the PI3KH1047R and empty vector expressing cells (Figure 1B). Finally, NeuT expressing MCF10A cells showed the least amount of autophagy induction following matrix detachment (Figure 1C). Although expression of oncogenes, particularly those that impinge on mTOR through the PI3K/AKT pathway, are thought to prevent autophagy induction, these initial results suggest that at least during matrix detachment autophagy can be induced in cells expressing oncogenes that activate the PI3K pathway.

B. *Express GFP-LC3 in MCF10A cell lines and measure levels of GFP-LC3 puncta following suspension:*

We have taken the above oncogene expressing MCF10A lines and stably introduced GFP-LC3 through retroviral infection. As detailed in the proposal, the induction of autophagy in cells expressing GFP-LC3 is visualized by an increase in GFP positive puncta within the cell. We used this technique to verify the findings described above in Tasks 1A and 1C. To accomplish this task, cells described above expressing GFP-LC3 were plated on poly-hema coated dishes for 24hrs. Cells were then fixed and mounted on coverslips and visualized by confocal microscopy. Since MCF10A cells clump together into tightly packed aggregates following suspension, we chose to use confocal microscopy hoping this would allow us to clearly visualize individual puncta within each cell as well as individual cells within aggregates. Images obtained from these experiments show the formation of GFP-LC3 puncta in the fixed cell aggregates (Figure 2). Unfortunately, we could not easily distinguish individual puncta or individual cells in the images we acquired due to cell-cell aggregation. Although, this

FIGURE 2

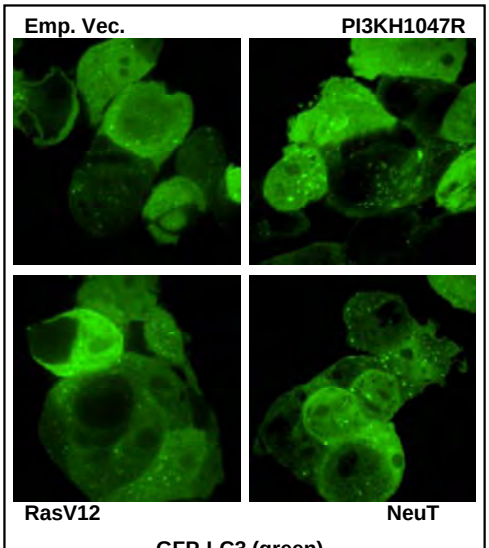

Figure 2: GFP-LC3 puncta in MCF10A cells expressing empty vector (Emp. Vec.) or PI3KH1047R, RasV12 or NeuT oncogenes following 24hrs of matrix detachment.

technical issue prevented us from quantifying the precise number of GFP-LC3 puncta per cell following matrix detachment, it is clear from the images in Figure 2 that autophagy is induced in oncogene expressing MCF10A cells during suspension and to a similar degree as empty vector controls.

D. *Stably co-express tandem mCherry-GFP LC3 in above cell lines.*

We created PI3KH1047R, NeuT and RasV12 MCF10A cells that stably express mCherry-GFP LC3. See Figure 3 for images showing expression.

E. *Examine LC3 turnover in mCherry-GFP-LC3 expressing cells by monitoring levels of GFP vs. Cherry positive puncta during multiple timepoints following suspension:*

FIGURE 3

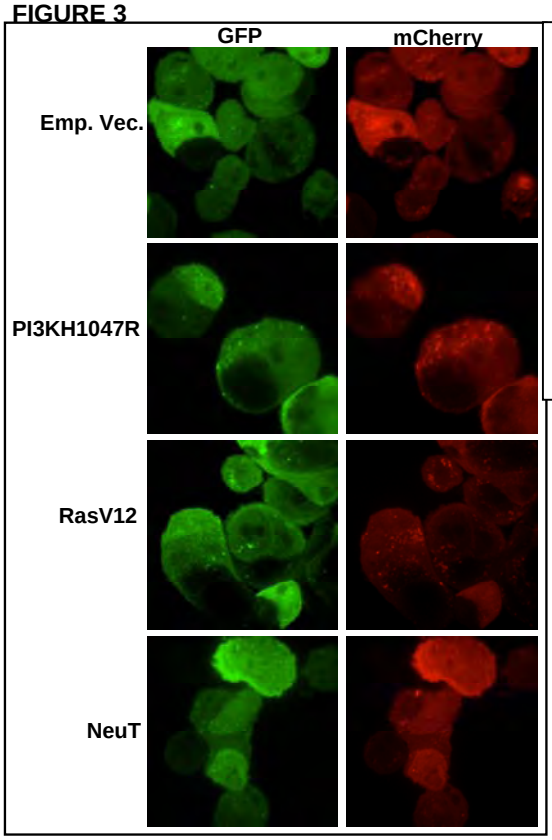

Figure 3: Images of mCherry-GFP-LC3 expressing MCF10A cell lines following 24hrs of suspension. Oncogene expressing cell lines (PI3KH1047R, RasV12 and NeuT) have similar levels of GFP and mCherry puncta as empty vector controls (Emp. Vec.). Each cell line also has a greater number of mCherry dots, which do not correspond to GFP dots on the adjacent image, indicating proper autophagic flux to the lysosome.

Stable cell lines expressing mCherry-GFP-LC3 were grown in suspension for up to 48hrs, mounted on coverslips, and imaged using confocal microscopy. We were able to visualize mCherry-GFP doubly positive puncta as well as mCherry only positive puncta in all the cell lines following suspension (Figure 3). As described in Task 2B, we encountered technical issues that prevented us from quantifying the number of single positive (mCherry only; corresponding to autophagosomes that have fused with the lysosome) and doubly positive puncta (both GFP and Cherry positive; early autophagosomes) per cell. First, due to the aggregates that form during suspension it was difficult to distinguish individual puncta as well as individual cells within cell aggregates. Second, we also noticed that levels of mCherry only puncta continued to increase at later timepoints during suspension and began to form larger puncta that looked liked aggregates. Due to the continued aggregation of mCherry during suspension, we were unable to reliably count the numbers of mCherry positive puncta versus mCherry-GFP double positive puncta. Even though we were unable to precisely quantify the number of puncta per cell, we could easily observe both single and double positive puncta in all cell lines during suspension. These results in combination with those from the previous tasks show that autophagy is induced in oncogene expressing MCF10A cells during suspension, and autophagosomes that form in these cells during suspension properly fuse and are degraded in the lysosome.

Task 2: *Determine if autophagy inhibition promotes the death of oncogene expressing cells during ECM detachment in both 2D and 3D models, and if autophagy contributes to oncogenic transformation.*

G: *Assess transformation potential of control, PI3KH1047R, and HER2/Neu expressing wild-type, ATG5 and ATG7 null MEFs:*

FIGURE 4

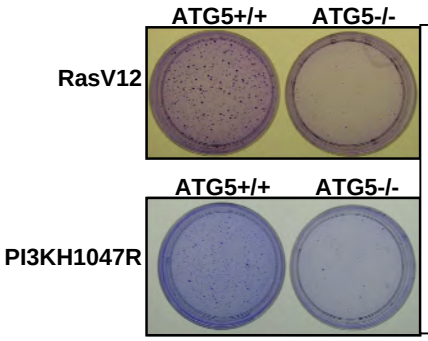

Figure 4: Representative soft agar plates comparing RasV12 expressing ATG5+/+ to RasV12 ATG5-/- MEFs (after 2 weeks of growth) and PI3KH1047R expressing ATG5+/+ to PI3KH1047R ATG5-/- MEFs (after 4 weeks of growth). Each oncogene is less transforming in the autophagy deficient (ATG5-/-) background, and RasV12 expression resulted in much more robust colony outgrowth.

In our original statement of work we proposed to first assess the contribution of autophagy to transformation potential using the MCF10A cell lines described in Task1 expressing short-hairpin RNAs (shRNAs) directed against different autophagy genes (ATGs). Due to the difficulties we encountered in achieving a high level of autophagy reduction using shRNAs, we decided to begin by examining transformation potential in autophagy deficient mouse embryonic fibroblasts (MEFs). We started by creating stable lines of wild-type, ATG5 knockout, and ATG7 knockout MEFs expressing PI3KH1047R and RasV12. We created RasV12 expressing lines as a positive control, as we were unsure about how efficiently PI3KH1047R expressing cells would grow in soft agar. Both RasV12 expressing as well as PI3KH1047R expressing MEFs were plated in soft agar and colonies were allowed to form for 2-4 weeks. In our initial experiments we compared the ability of RasV12 and PI3KH1047R wild-type MEFs to form colonies in soft agar compared to RasV12 and PI3KH1047R ATG5 knockout MEFs. With both the RasV12 and the PI3KH1047R expressing cell lines we saw a noticeable decrease in the colony number in the ATG5 deficient background (Figure 4). The RasV12 expressing cells formed large colonies following 2 weeks of growth, however, colonies that formed in the PI3KH1047R lines were much smaller and took 4 weeks to grow out. Due to the

FIGURE 5

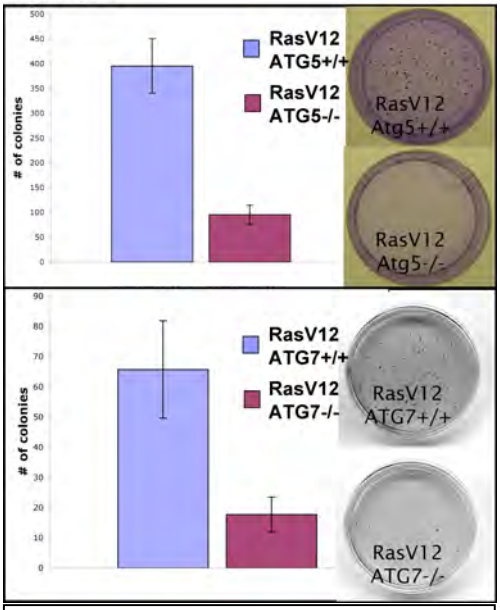

Figure 5: Soft agar transformation of RasV12 expressing ATG5+/+ versus ATG5-/- MEFs and RasV12 expressing ATG7+/+ versus ATG7-/- MEFs. Colony numbers are displayed in the bar graphs on the left.
ATG5 experiment; $p<0.00002$.
ATG7 experiment; $p<0.0004$

poor transformation ability of PI3KH1047R in these cell lines and the difference that we observed in colony outgrowth between the RasV12 ATG5 wild-type and knockout cells, we decided to use these cells to assess the contribution of autophagy during soft agar colony formation. Although, activated Ras mutations are not commonly found in breast cancer, they arrise in some of the most aggressive breast cancers and are present in some of the breast cancer cell lines we propose to use in Task 3. We repeated this assay multiple times in the RasV12 wild-type versus RasV12 ATG5 knockout MEFs as well as in wild-type and ATG7 knockout MEFs expressing RasV12. Images of the soft agar plates as well as quantification of the number of colonies can be seen in Figure 5. In both the ATG7 and ATG5 knockout background we saw a large decrease in colony number when compared to wild-type controls.

2A: *Optimize siRNA transfection and validate knockdown of ATG5 and ATG7 in empty vector, PI3KH1047R, and HER2/NEU MCF10A cells.*

Based on the robust transformation phenotype we achieved with RasV12 expression in MEFs, we decided to first examine transformation and 3D morphogenesis in RasV12 expressing MCF10A cells. For siRNA validation, we tested siRNAs against ATG12 and ATG7 in RasV12 and empty vector MCF10A cells. We chose to use siRNA against ATG12 instead of ATG5 due to availability. Since ATG12 and ATG5 form a constitutive, stable complex that is necessary for autophagy induction, knockdown of either gene is sufficient to reduce levels of the ATG12:ATG5 complex. Although we were able to visualize some level of knockdown of the intended targets with the siRNAs that we tried, they did not cause a significant reduction in the levels of autophagy (data not shown). This suggests that the residual levels of protein present following siRNA transfection were sufficient to maximally induce the autophagic process. Since we were unable to significantly reduce the levels of autophagy with these siATGs, we were unable to use this method of knockdown for subsequent experiments. However, we were able to validate both knockdown and a reduction in autophagy following expression of a short-hairpin RNA (shRNA) against ATG7 that is described below. We plan on using this shRNA in Tasks 2C and D in which we previously planned on using siRNAs.

2B: *Stably express short hairpin RNAs against ATG5 and ATG7 in empty vector, PI3KH1047R, and HER2/NEU MCF10A cells and validate knockdown by western blot.*

We tested multiple short hairpin RNAs (shRNAs) against several ATG genes, and found shRNAs directed against ATG7 (Figure 6) and ATG12 (data not shown) resulted in a high level of target knockdown when stably expressed in the MCF10A cell lines. We achieved the best results with a hairpin directed against ATG7, which can be seen in Figure 6. Expression of this hairpin in empty vector and RasV12 expressing MCF10A cells consistently

FIGURE 6

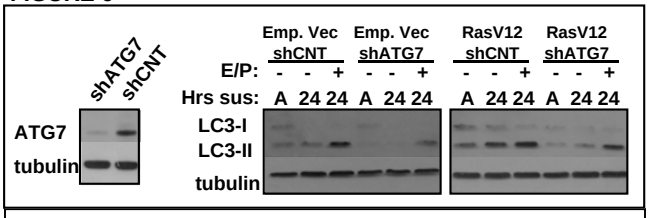

Figure 6: Knockdown levels of ATG7 and resulting autophagy levels in MCF10A cells expressing shATG7. The blot on the left shows ATG7 knockdown levels achieved in MCF10A cells following expression of shATG7. Blots to the right show resulting autophagy (LC3-II) reduction in empty vector (Emp. Vec.) and RasV12 expressing MCF10A cells following 24 hrs of suspension (24) or in attached conditions (A) either with (+) or without (-) E64d and pepstatin A (E/P).

results in a high level of ATG7 knockdown as well as a noticeable reduction in the levels of autophagy induction. Although we were unable to completely inhibit autophagy levels by expressing shATG7 in the MCF10A cell lines, the reduction of autophagy we are able to achieve is better than what was seen in siRNA transfected cells. Interestingly, in testing these hairpins, we noticed that several shRNAs against ATGs were toxic to the RasV12 expressing MCF10A cells but not to empty vector control cells. This observation suggests that the added stress of an oncogene in the MCF10A cells may require autophagy for sustained growth or to mitigate cytotoxic damage that results from oncogenic stress.

2C: *Measure levels of apoptosis in above cells transfected with siATGs versus siControl:*

As described in Task 2A, we experienced technical difficulties trying to reduce autophagy by knocking down ATGs using siRNA. Instead we plan on using the shRNAs against ATGs described in the previous task.

2D: *Validate differences observed in apoptosis by clonogenic viability assay:*

As described for Task 2C we have not yet started the apoptosis experiments due to difficulties we encountered in achieving sufficient knockdown using siRNAs. We will perform this experiment using the shRNAs against ATGs that are described in Task 2B.

2E: *Set-up 3D morphogenesis assays in the MCF10A cell lines described above:*

We have started to evaluate the contribution of autophagy during 3D morphogenesis of oncogene expressing MCF10A cells by utilizing the MCF10A expressing RasV12 and shATG7 described in Task 2B. When grown on a laminin rich basement membrane, MCF10A cells are able to form polarized acini with a hollow lumen that recapitulate many aspects of glands *in vivo* (Debnath et al., 2005). The hollowing of the lumen in these structures occurs from the selective apoptosis of the inner cells and we have previously found that inhibition of autophagy increases the rate of lumen clearance (Fung et al., 2007). When RasV12 expressing MCF10As are grown in 3D, polarity and growth suppression is lost resulting in the formation of branched, invasive structures. We originally sought to determine if the increase levels of apoptosis we saw in wild-type MCF10As expressing shRNA against ATGs would also occur in oncogene expressing MCF10A cells. Interestingly, when we inhibited autophagy in RasV12 expressing MCF10A cells during 3D morphogenesis, we observed not only a decrease in the overall size and number of structures that formed, but also an inhibition of the branching-invasive phenotype seen in shControl expressing cells (Figure 7). First, since we observed an overall decrease in structure size and number in cells expressing shATG7, we isolated cells on day 10 of growth and counted the total number of cells per well. We found that there were approximately three times more cells in the shControl wells compared to the shATG7 wells ($30.3x10^4$ versus $8.7x10^4$, respectively) on day 10 of growth suggesting either an increase in proliferation or a decrease in the level of apoptosis in the shControl structures. To detect

FIGURE 7

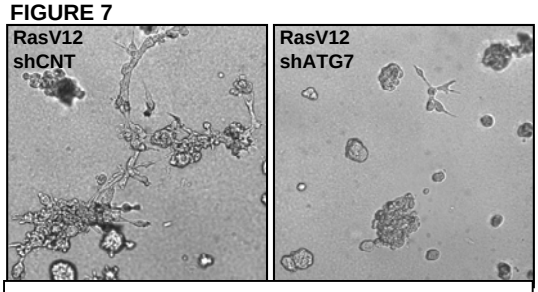

Figure7: Low power phase images of 3D structures formed by RasV12 shCNT and RasV12 shATG7 MCF10A cells. Note the shATG7 expressing cells form smaller structures with decreased branching.

proliferation levels, we stained the structures with Ki67, a marker for cells in cycle, and observed a decrease in the number of structures in RasV12 shATG7 wells that were Ki67 positive (data not shown). This indicates a decrease in proliferation in RasV12 MCF10A cells expressing shATG7.

As mentioned above, in addition to the decrease in the number and the size of the structures that grew out in RasV12 MCF10As expressing shATG7, we also noticed that the structures lacked the branching phenotype that occurs upon RasV12 expression. Upon staining the structures with cell-cell junction and polarity markers (beta-catenin and laminin V), we noticed that there was a partial restoration in both cell-cell contacts and basement membrane polarity in many of the RasV12 shATG7 expressing structures (Figure 8). The acquisition of an invasive phenotype is a crucial step in the progression of breast cancer towards metastatic disease. We believe this finding could have important implications in understanding how breast cancer cells acquire invasive characteristics that allow them to disseminate from the primary tumor. We plan on studying this finding in more detail by looking at specific markers that contribute to increased invasive potential, such as the loss of E-cadherin. We will examine how autophagy inhibition can modulate these markers in oncogene expressing cells and how autophagy contributes to the enhanced invasiveness that occurs during oncogenic transformation.

FIGURE 8

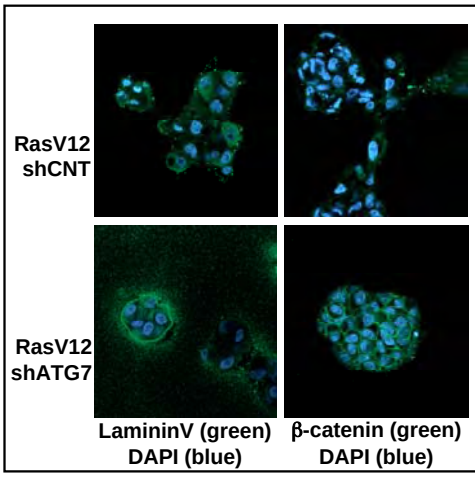

Figure 8: Images showing expression of polarity (lamininV) and cell-cell contact markers (β-catenin) in green and nuclei (DAPI) in blue in 3D structures formed by RasV12 shCNT and RasV12 shATG7 MCF10A cells. Note the restoration of basally expressed lamininV in the bottom left panel and expression of β-catenin between cells in the bottom right panel in shATG7 expressing cells.

2F: *Measure apoptosis (cleaved-caspase 3 staining) in 3D structures:*

As described in Task 2E, we have previously found that wild-type MCF10As expressing shRNAs against various ATGs have increased levels of apoptosis during 3D morphogenesis. We therefore decided to determine if this held true for oncogene expressing MCF10A cells grown in 3D. To date, we have examined the levels of cleaved-caspase 3 staining in the RasV12 MCF10A shControl structures and RasV12 MCF10A shATG7 structures described in the previous task. Expression of RasV12 in MCF10A has been previously shown to suppress apoptosis induced by matrix detachment (Reginato et al., 2003). In line with this, we saw very little apoptosis in RasV12 MCF10A shCNT structures. We were able to visualize occasional cleaved-caspase 3 positive cells, but typically only a few per structure, and these positive cells did not necessarily correspond to cells located internally. We observed similar levels of apoptosis RasV12 MCF10A expressing shATG7 (data not shown). This suggests that autophagy inhibition is not sufficient to overcome the apoptotic suppression that results from oncogene expression in MCF10A cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Western blot analysis of autophagy induction following matrix detachment of oncogene expressing MCF10A cells
- Confirmation of autophagy induction in oncogene expressing MCF10A cells by examination of both GFP-LC3 puncta and mCherry-GFP-LC3 puncta following matrix detachment.
- Testing and verifying short hairpins against ATGs in MCF10A cell lines
- Characterization of 3D morphogenesis phenotype resulting from autophagy inhibition in RasV12 expressing MCF10A cells
- Evaluation of apoptosis levels during 3D morphogenesis in RasV12 MCF10A cells expressing shATG7

REPORTABLE OUTCOMES:

Publications:

Lock, R. and Debnath, J. **Extracellular matrix regulation of autophagy.** Curr Opin Cell Biol. 2008 Oct;20(5):583-8.

CONCLUSION:

Since the funding of this award, we have determined that autophagy is induced in breast epithelial cells expressing oncogenes that activate the PI3K and Ras pathways both by western blot analysis of LC3-II levels and by visualization of GFP-LC3 and mCherry-GFP-LC3 puncta formation. This result is important in understanding the role of autophagy in breast cancer development as it has been assumed that activating mutations in the PI3K and Ras pathways are sufficient to suppress autophagy induction. Next, we have validated short-hairpin RNAs against autophagy genes that are able to not only achieve knockdown of the intended targets, but also significantly inhibit autophagy induction. These shRNAs are critical to evaluating the contribution of autophagy during 3D morphogenesis and transformation in oncogene expressing MCF10A cells as well as the breast cancer cell lines we intend to use in Task 3. Finally, we have begun to examine the role of autophagy during 3D morphogenesis of oncogene expressing breast epithelial cells using RasV12 MCF10A cells expressing shATG7. Although autophagy inhibition did not cause an increase in the levels of apoptosis in the structures, as originally hypothesized, we found that autophagy inhibition was sufficient to decrease both the growth and invasiveness of RasV12 MCF10A structures. Importantly, the decreased invasiveness seen in the RasV12 MCF10A structures expressing shATG7 corresponded with a partial restoration in polarity and cell-cell contacts. These preliminary results are significant as it points to a potential role for autophagy in contributing to invasive and metastatic potential during tumorigenesis. These results will contribute to a better understanding of how autophagy might be modulated during breast cancer development and how it might enhance breast cancer progression.

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APPENDICES:

None.

SUPPORTING DATA:

Embedded in text.